

UTILITY PATENT APPLICATION TRANSMITTAL (Small Entity)

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Docket No.

2019659 - 0139

Total Pages in this Submission

TO THE ASSISTANT COMMISSIONER FOR PATENTS

Box Patent Application
Washington, D.C. 20231

Transmitted herewith for filing under 35 U.S.C. 111(a) and 37 C.F.R. 1.53(b) is a new utility patent application for an invention entitled:

METHOD FOR TREATING RESPIRATORY DISTRESS SYNDROME

and invented by:

Vicky L. Funanage, Sandra G. Hassinnk, Susan M. Kirwin, and Darlise O'Connor

If a **CONTINUATION APPLICATION**, check appropriate box and supply the requisite information:

☐ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No.: _____

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Enclosed are:

Application Elements

1. ☒ Filing fee as calculated and transmitted as described below
2. ☒ Specification having 25 pages and including the following:
 - a. ☒ Descriptive Title of the Invention
 - b. ☐ Cross References to Related Applications (if applicable)
 - c. ☐ Statement Regarding Federally-sponsored Research/Development (if applicable)
 - d. ☐ Reference to Microfiche Appendix (if applicable)
 - e. ☒ Background of the Invention
 - f. ☒ Brief Summary of the Invention
 - g. ☒ Brief Description of the Drawings (if drawings filed)
 - h. ☒ Detailed Description
 - i. ☒ Claim(s) as Classified Below
 - j. ☒ Abstract of the Disclosure

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Application Elements (Continued)

3. ☒ Drawing(s) (when necessary as prescribed by 35 USC 113)
- a. ☒ Formal b. ☐ Informal Number of Sheets 6
4. ☒ Oath or Declaration
- a. ☒ Newly executed (original or copy) ☐ Unexecuted
- b. ☐ Copy from a prior application (37 CFR 1.63(d)) (for continuation/divisional application only)
- c. ☒ With Power of Attorney ☐ Without Power of Attorney
- d. ☐ DELETION OF INVENTOR(S)
Signed statement attached deleting inventor(s) named in the prior application,
see 37 C.F.R. 1.63(d)(2) and 1.33(b).
5. ☐ Incorporation By Reference (usable if Box 4b is checked)
The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.
6. ☐ Computer Program in Microfiche
7. ☐ Genetic Sequence Submission (if applicable, all must be included)
- a. ☐ Paper Copy
- b. ☐ Computer Readable Copy
- c. ☐ Statement Verifying Identical Paper and Computer Readable Copy

Accompanying Application Parts

8. ☒ Assignment Papers (cover sheet & documents)
9. ☐ 37 CFR 3.73(b) Statement (when there is an assignee)
10. ☐ English Translation Document (if applicable)
11. ☐ Information Disclosure Statement/PTO-1449 ☐ Copies of IDS Citations
12. ☐ Preliminary Amendment
13. ☒ Acknowledgment postcard
14. ☐ Certificate of Mailing
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Accompanying Application Parts (Continued)

15. ☐ Certified Copy of Priority Document(s) *(if foreign priority is claimed)*
16. ☒ Small Entity Statement(s) - Specify Number of Statements Submitted: one
17. ☐ Additional Enclosures *(please identify below):*

Request That Application Not Be Published Pursuant To 35 U.S.C. 122(b)(2)

18. ☐ Pursuant to 35 U.S.C. 122(b)(2), Applicant hereby requests that this patent application not be published pursuant to 35 U.S.C. 122(b)(1). Applicant hereby certifies that the invention disclosed in this application has not and will not be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication of applications 18 months after filing of the application.

Warning

An applicant who makes a request not to publish, but who subsequently files in a foreign country or under a multilateral international agreement specified in 35 U.S.C. 122(b)(2)(B)(i), must notify the Director of such filing not later than 45 days after the date of the filing of such foreign or international application. A failure of the applicant to provide such notice within the prescribed period shall result in the application being regarded as abandoned, unless it is shown to the satisfaction of the Director that the delay in submitting the notice was unintentional.

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Fee Calculation and Transmittal

CLAIMS AS FILED

For	#Filed	#Allowed	#Extra	Rate	Fee
Total Claims	16	- 20 =	0	x \$9.00	\$0.00
Indep. Claims	3	- 3 =	0	x \$40.00	\$0.00
Multiple Dependent Claims (check if applicable) <input type="checkbox"/>					\$0.00
BASIC FEE					\$355.00
OTHER FEE (specify purpose) _____					\$0.00
TOTAL FILING FEE					\$355.00

- ☒ A check in the amount of \$355.00 to cover the filing fee is enclosed.
- ☒ The Commissioner is hereby authorized to charge and credit Deposit Account No. 23-1951 as described below. A duplicate copy of this sheet is enclosed.
- ☐ Charge the amount of _____ as filing fee.
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- ☒ Charge any additional filing fees required under 37 C.F.R. 1.16 and 1.17.
- ☐ Charge the issue fee set in 37 C.F.R. 1.18 at the mailing of the Notice of Allowance, pursuant to 37 C.F.R. 1.311(b).

Dated: November 14, 2000



Signature

Philip D. Lane
Registration No. 41,140
McGuireWoods, LLP
1750 Tysons Blvd, Suite 1800
McLean, Virginia 22102

CC:

703-712-5069

**VERIFIED STATEMENT (DECLARATION) CLAIMING
SMALL ENTITY STATUS****(37 C.F.R. §§1.9(f) and 1.27 (d)) - NONPROFIT ORGANIZATION**

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION The Nemours Foundation

ADDRESS OF ORGANIZATION 1600 Rockland Road, Wilmington, Delaware 19899

TYPE OF ORGANIZATION

- (check one) ☐ UNIVERSITY OR OTHER INSTITUTION OF HIGHER LEARNING
- ☒ TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3))
- ☐ NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA
(NAME OF STATE _____)
(CITATION OF STATUTE _____)
- ☐ WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3)) IF LOCATED IN UNITED STATES OF AMERICA
- ☐ WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA
(NAME OF STATE _____)
(CITATION OF STATUTE _____)

I hereby declare that the above identified nonprofit organization qualifies as a nonprofit organization as defined in 37 C.F.R. §1.9(e), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, with regard to the invention entitled METHOD FOR TREATING RESPIRATORY DISTRESS SYNDROME by inventors Vicky L. Funanage, et al. described in

- (check one) ☒ the specification filed herewith.
- ☐ application Serial No. _____ filed _____
- ☐ Patent No. 5, _____, issued _____, 199__.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the above identified invention.

If the rights held by the above identified nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 C.F.R. §1.9(d) or by any concern which would not qualify as a small business concern under 37 C.F.R. §1.9(d) or a nonprofit organization under 37 C.F.R. §1.9(e). *NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 C.F.R. §1.27)

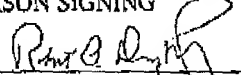
Page 2 of 2

NAME _____
ADDRESS _____
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NAME _____
ADDRESS _____
☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 C.F.R. §1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Robert A. Doughty, M.D., Ph.D.
TITLE OF PERSON IN ORGANIZATION Vice President of Physician Practices
ADDRESS OF PERSON SIGNING 1650 Prudential Drive, Suite 300,
Jacksonville, Florida 32207
SIGNATURE  DATE 11/14/00

Method For Treating Respiratory Distress Syndrome

BACKGROUND

I. Field of Invention

The present invention provides a new method for treating pulmonary distress caused by low or insufficient surfactant production in infants, children or adults. More particularly, the method of the invention utilizes leptin to treat individuals with impaired lung surfactant production and is particularly useful for treating Respiratory Distress Syndrome (RDS) in premature infants.

II. Background of Invention

Premature infants are at increased risk for developing Respiratory Distress Syndrome (RDS), the leading cause of neonatal morbidity and mortality in developed countries (Mendelson et al., Bailliere's Clin. Endocrinol. Metab. 4:351-78, 1990). This condition is caused by a deficiency of lung surfactant, a complex material consisting of phospholipids, neutral lipids, carbohydrate and proteins. These infants require assisted ventilation and supplemental oxygen for prolonged periods of time. Often these infants develop Bronchopulmonary Dysplasia (BPD), a chronic lung disease associated with neurodevelopmental delay, poor growth, and late mortality (Bader et al., J. Pediatr. 110(5):693-9, 1987; Gibson et al., Am. J. Dis. Child. 143(7):721-5, 1988; Kurzner et al., Pediatrics 81(3):379-84, 1988; Vohr et al., Dev. Med. Child. Neurol. 33(8):690-7, 1991). Inflammation, primarily due to oxygen-induced free radical formation, positive pressure ventilation, and infection, is thought to be a key factor in the lung injury observed in these infants (Pierce and Bancalari, Pediatr. Pulmonol. 19(6):371-8, 1995).

Strategies aimed at treating the pulmonary inflammation in BPD through the use of systemic steroids have not shown a favorable outcome in decreasing the overall incidence of this disease. Three large multicenter trials, which enrolled a total of 1348 infants, independently showed no significant benefit to early administration (within 72 hours of life) of steroids on the incidence of BPD (Halliday et al., In: Hot Topics in Neonatology, Ross Laboratories, pp. 267-75, 1999; Soll et al., *Pediatr. Res.* 45: 226A, 1999; Stark et al., *Pediatrics Supplement* 104: 739A, 1999). Two of these trials were stopped prematurely due to concerns of significant detrimental side effects, including gastrointestinal perforation, periventricular leukomalacia, poor weight gain, gastrointestinal hemorrhage, and hypertension. Long term follow-up studies have shown a significant detrimental effect on somatic growth (Gibson et al., *Arch. Dis. Child* 69: 505-9, 1993, Yeh et al., *Pediatrics* 101(5):E7, 1998; O'Shea et al., *Pediatrics* 104(1 part 1):15-21, 1999). These adverse effects may be related to the catabolic effects of steroids on growing tissues (Tsai et al., *Act. Paediatr.* 85(12):1487-90, 1996). Efforts to reduce the incidence of BPD using other strategies such as inhaled steroids, high-frequency ventilation, and treatment of RDS with surfactant have also shown mixed results. There has been some success in reducing the incidence of RDS by enhancing surfactant production in utero via glucocorticoid administration to mothers in preterm labor (Liggins and Howie, *Pediatrics* 59:515-25, 1972). However, this treatment strategy is dependent on accurate identification of those mothers at risk for preterm delivery, and thus, is only effective for a small subset of premature infants affected with RDS. Furthermore, despite significant advances in neonatal care during the past three decades, the incidence of RDS and BPD has not changed significantly. There remains a clear need to identify alternative treatment strategies for this disease.

As more fully described below, the present invention overcomes the problems associated with previous forms of RDS therapy and includes a novel method of treating RDS that can be administered to premature infants as well as infants, children or adult subjects who have deficient lung surfactant.

Summary of the Invention

The present invention includes a method for treating respiratory distress by treatment with leptin. According to the invention, leptin may be administered orally as well as by intravenously, intramuscularly, and other parenteral and enteral means. In one embodiment, the invention includes a method for treating RDS and BPD in premature infants. Another aspect of this invention is its usefulness for treating infants, children or adults suffering from pulmonary distress caused by low or insufficient production of surfactant. The present invention overcomes the problems associated with previous forms of RDS therapy, particularly the use of steroids.

The method of the invention provides for improving lung surfactant production in an individual with impaired surfactant production by administering a leptin compound to the individual for a time and in an amount sufficient to enhance surfactant production. The individual may be any mammal. Further, while the invention may be used for the treatment of any individual with impaired lung surfactant production, it is particularly useful for treating infants with intrauterine development of less than nine months. The leptin compound may comprise at least a biologically active fragment of leptin that is capable of binding to the leptin receptor and eliciting a biological effect such as increased surfactant production, and may be derived from any source of leptin or a biologically active fragment of leptin including recombinant protein.

In the method of the invention, the leptin compound is administered in a dosage from

about 0.1 ng/kg body weight to about 100 mg/kg body weight and by a method selected from the group consisting of subcutaneously, intradermally, intravenously, intramuscularly, intraperitoneally, transdermally, orally, enteral tube feeding, pulmonary delivery, intranasal delivery, controlled release delivery and pump delivery.

5 Leptin may be administered with nutritional supplements, growth factors, and steroids, such as dexamethasone, that increase lung function. In a preferred embodiment, the growth factors may be selected from the group consisting of epidermal growth factor, fibroblast growth factor, insulin-like growth factor, thyroid hormone, and platelet derived growth factor.

Further, the method of the invention which comprises administering a leptin compound
10 to an individual with impaired lung surfactant production for a time and in an amount sufficient to enhance surfactant production is particularly suitable for the treatment of individuals with Respiratory Distress Syndrome (RDS) and/or Bronchopulmonary Dysplasia (BPD). For treating RDS or BPD, the leptin compound comprises at least a biologically active fragment of leptin which is administered in a dosage from about 0.1 ng/kg body weight to about 100 mg/kg body
15 weight. The leptin compound is administered by a method selected from the group consisting of subcutaneously, intradermally, intravenously, intramuscularly, intraperitoneally, transdermally, orally, enteral tube feeding, pulmonary delivery, intranasal delivery, controlled release delivery and pump delivery.

Brief Description of the Figures

20 Figure 1 is a Western blot analysis of SP-B (surfactant protein-B) from 21-day gestation, fetal rat lung explants cultured in the presence or absence of leptin. Fetal lung explants were exposed to either (A) 1 ng/ml or (B) 10 ng/ml leptin from initiation of culture (day 0). Explants

were harvested and protein extracts were prepared on days (d) 1, 2, and 3 of culture. Western blots were probed with an antibody specific to rat SP-B.

Figure 2 shows RT-PCR analysis of surfactant protein A (SP-A), surfactant protein B (SP-B), surfactant protein C (SP-C), and β -actin mRNA expression in day 17 fetal lung explant cultures. Cultures were exposed to 1 ng/ml leptin (lanes 3, A-C) or control medium (lanes 2, A-C), and total RNA was isolated at the indicated day of culture. Total RNA was reverse transcribed and amplified with both β -actin and leptin PCR primers. In each figure, lane 1 is the time of initiation of culture (day 0). Low levels of all surfactant RNAs were detected in the uncultured cells (lanes 1, A-C). The size of the β -actin RT/PCR product is 492 bp; the size of SP-A is 352 bp; SP-B is 201 bp, and SP-C is 284 bp.

Figure 3 shows RT-PCR results of an experiment that determines the relative levels of the surfactant mRNAs to that of 18S rRNA in day 17 fetal lung explants. Qualitative RT/PCR detection of surfactant mRNA was performed using the Ambion QuantumRNA kit (Ambion, Austin, TX) and 1 μ g of total RNA isolated from explants cultured for 3 days in culture. Lanes 1, 3,5: control day 3; lanes 2, 4, 6: 1 ng/ml leptin day 3.

Figure 4 depicts RT-PCR analysis of leptin receptor expression in day 17 fetal lung explant cultures. Total RNA was reverse transcribed and amplified with either the short (OB-Ra) or the long (OB-Rb) form of the leptin receptor. Day 0 (Cd0) represents the time of initiation of culture. The fetal lung explants were exposed for 3 days to either control medium (Cd3), 1 ng/ml leptin (Ld3), or 10 nM dexamethasone (Dexd3). The size of the OB-Ra RT/PCR product is 479 bp and OB-Rb is 262 bp.

Figure 5 depicts RT-PCR analysis of surfactant and GADPH (glyceraldehyde phosphate dehydrogenase) mRNA expression in cultured isolated type II alveolar cells. Cells were exposed to either 1 ng/ml leptin (1L) or 10 ng/ml leptin (10L). Control cells were not exposed to leptin.

Figure 6 depicts the effect of antenatal treatment with leptin or dexamethasone on fetal lung morphology. Fetal lung sections were stained with a histochemical stain for alkaline phosphatase, which identifies type II cells. Control, no leptin; dexamethasone (6 mg/ml for 48 h); leptin (0.25 mg/ml for 48 h).

Detailed Description of the Invention

The present invention includes a method for treating RDS by treatment with leptin. The invention is based on studies that show that leptin is produced by the placenta, that leptin levels in cord blood are correlated with newborn birth weights, that leptin is produced by the mammary gland and is found in breast milk, that leptin levels are higher in female as compared to male newborns, and that leptin augments surfactant production in fetal lung explant cultures. The combination of this role in fetal lung development with the known facts that the incidence of RDS mortality is lower in female as opposed to male infants and lower in breast-fed than in formula-fed infants points to an important role of leptin in maturation and function of the lung and forms the basis of the method of this invention.

Recent studies on the hormone leptin have outlined its role in energy homeostasis, regulating such diverse processes as satiety, fetal and neonatal growth, and immune function (reviewed in Campfield, Smith, and Burn, Science 280: 1383-7, 1998). Leptin, a 167 amino acid cytokine hormone produced by the obesity (*ob*) gene, was initially thought to be adipocyte-specific (Zhang et al., Nature 372: 425-32, 1994). However, Hassink et al. (Pediatrics 100: e1-e6, 7, 1997) have discovered high level expression of leptin mRNA and protein in human

placenta, and speculated that leptin was produced by the syncytiotrophoblasts. This was subsequently confirmed by Masuzaki et al. (Nat. Med. 3:1029-33, 1997). Additionally, leptin has been shown to be produced by gastric epithelium (Bado et al., J. Clin. Endo. Metab. 82: 1642-5, 1998) and the mammary gland (Smith-Kirwin et al., J. Clin. Endo. Metab. 83: 1810-3, 1998). Furthermore, under conditions of nutrient deprivation, leptin is also produced in skeletal muscle and induces its own expression in this tissue (Wang et al., Nature 393: 684-8, 1998; Nat. Med 5: 895-9, 1999).

Leptin regulates appetite and metabolic activity in mice (Rohner-Jeanreanaud and Jeanreanaud, N. Engl. J. Med. 344: 324-5, 1996) by acting through the long form of the leptin receptor (OB-Rb) in the hypothalamus (Campfield, Smith, and Burn, Science 280: 1383-7, 1998). Recently, additional roles for leptin have been suggested. Leptin has been demonstrated to have angiogenic activity in vivo and in vitro (Sierra-Honigsmann et al., Science 281: 1683-6, 1988). These studies showed that leptin induces neovascularization in cornea from normal rats but not fa/fa Zucker rats, which lack a functional leptin receptor. In leptin-deficient (*ob/ob*) mice, puberty and pregnancy cannot be established without leptin administration (Chehab, Lim, and Lu, Nature Gen. 12: 318-20, 1996), indicating that leptin may have a role in sexual maturation and development (Hassink et al., Pediatrics 98: 201-5, 1996). Other roles for leptin include a regulator of hematopoiesis (Cioffi et al., Nature Medicine 2: 585-9, 1996; Gainsford et al., Proc. Natl. Acad. Sci. USA 93: 14564-8, 1996), glucose metabolism (Kamohara et al., Nature 389: 374-7, 1997), and proinflammatory immune responses (Loffreda et al., FASEB J. 12: 57-65, 1998; Lord et al., Nature 394: 897-901, 1998). Leptin-deficient *ob/ob* mice were also noted to have a specific respiratory phenotype of alveolar hyperventilation and chronic hypercapnia (Tankersley et al., J. Appl. Physiol. 81: 716-23, 1996). These phenotypic abnormalities were

noted in age and weight-matched *ob/ob* mice before pronounced obesity. In a subsequent study, Tankersley et al. (J. Appl. Physiol. 85: 2261-9, 1998) demonstrated that leptin administration to *ob/ob* mice ameliorated the volume-dependent decrease in lung compliance in these animals. leptin has also recently been shown to prevent respiratory depression in *ob/ob* mice (O'Donnell et al., Am. J. Resp. Crit. Care Med. 159: 1477-84, 1999). Because these *ob/ob* mice were born to either wild type or *ob/+* mothers, the fetuses were exposed to placental and possibly, maternal, leptin in utero. Thus, it was not possible to ascertain whether leptin affects fetal lung development in utero.

The observation that leptin is synthesized and secreted by human (Hassink et al., Pediatrics 100: e1-e6, 1997), rat (Chien et al., Biochem. Biophys. Commun. 237: 476-80, 1997) and mouse (Hoggard et al., Proc Nat Acad Sci. 94: 1073-8, 1997) placental tissue has important implications, since it suggests a novel role for leptin in fetal growth and development. Because leptin expression was observed in human placenta from near-term pregnancies and in mouse placenta from 14.5 day but not from 12 day of gestation (Tomimatsui et al., Biochem. Biophys. Res. Comm. 240:213-5, 1997; Hoggard et al., Proc. Nat. Acad. Sci. 94:1073-8, 1997), it is suggested that leptin regulates some aspect of developmental growth in the fetus during the second half of gestation. In the human, leptin has been detected in cord blood as early as 18 weeks of gestation (Jaquet et al., J. Clin. Endo. Metab. 83:1243-6, 1998). Leptin levels in cord blood increase with gestation (Jaquet et al., J. Clin. Endo. Metab. 83:1243-6, 1998) and show a good correlation with the birth weight of the newborn (Hassink et al., Pediatrics 100:e1-e6, 1997; Matsuda et al., J. Clin. Endo. Metab. 82:1642-4, 1997), further supporting the hypothesis that leptin regulates fetal growth. Premature infants are delivered before the late pregnancy rise in

leptin occurs (Masuzaki et al., Nat. Med. 3:1029-33, 1997) and have low cord blood leptin levels (Highman et al., Am. J. Obstet. Gynecol. 178:1010-5, 1998).

Without intending to be bound by theory, it is believed that leptin is important for lung growth and/or maturation and that the lack of leptin exposure late in pregnancy when the type II alveolar cells are maturing and producing surfactant could contribute to the respiratory distress suffered by many premature infants. Since leptin has been found in amniotic fluid, leptin may have a direct effect on type II alveolar cell maturation and growth, thereby increasing surfactant production.

The inadequacy and/or absence of pulmonary surfactant production at birth is one of the most serious and life threatening problems faced by the premature infant. Surfactant production is a maturation-dependent process, and deficiency results in RDS, which is characterized by inability to expand the alveoli and sustain adequate ventilation. Currently, treatment of RDS involves administering prenatal steroids to the mother in an attempt to increase surfactant production prior to delivery, administration of exogenous surfactant to the premature infant after birth, and supportive treatment with artificial ventilation until the premature infant's lungs mature. RDS accounts for a substantial burden of morbidity and mortality in premature infants, as well as significant emotional and financial burdens on the family. Although steroids increase serum leptin levels, there is recent evidence that glucocorticoids interfere with leptin's interaction with its receptor (Ur et al., Horm. Metab. Res. 28:4744-7, 1996; Zakrzewska et al., Diabetes 46:717-9, 1997). Furthermore, glucocorticoids may contribute to the development of central leptin resistance (Zakrzewska et al., Diabetes 46:717-9, 1997). These effects of glucocorticoids may contribute to the poor growth observed in premature infants treated with

steroids. Therefore, it is desirable to provide a method for treating RDS that does not involve the use of steroids.

The method of the present invention, which consists of administration of a leptin compound to a patient, avoids the use of steroids while providing effective treatment for premature infants who suffer from conditions in which there is insufficient production of surfactant. The method is also effective for treatment of newborns, infants, children, and adults who suffer from any condition caused by insufficient surfactant production as administration of the leptin compound is expected to increase surfactant production and improve lung function.

More particularly, the present invention provides a method for restoring pulmonary function in a patient who suffers from a lung disease characterized by insufficient surfactant production. Abnormalities of surfactant production have been described in obstructive lung diseases, such as asthma, bronchitis, chronic obstructive pulmonary disease, and following lung transplantation (reviewed in Grise, Eur. Resp. J. 13(6): 1455-76, 1999). Abnormal surfactant production has also been seen in infectious and suppurative lung diseases, such as cystic fibrosis, pneumonia, and AIDS. Finally, insufficient surfactant also characterizes diseases such as acute respiratory distress syndrome (ARDS), pulmonary edema, interstitial lung diseases, pulmonary alveolar proteinosis, following cardiopulmonary bypass, and in smokers. The method comprises administering a leptin compound to the host for a time and in an amount sufficient to restore or enhance respiratory function. Typically, the leptin compound will be administered in a dosage from about 0.1 ng per kg body weight to about 100 mg per kg body weight, for example. Effective amounts are determined by such factors as the leptin composition, the mode of administration, the weight and general health of the patient, and the judgment of the prescribing

physician, for example. Considerations associated with such factors are well known by those persons skilled in the art.

Further, the present invention, which has been discussed in the context of human patients, is not limited to use in humans, but is also effective in treating respiratory conditions caused by

5 inadequate surfactant production in other mammalian species

In the treatment of premature infants, the leptin compound is typically administered to any premature infant at increased risk of developing RDS from the onset of birth to the time when the infant would have reached full gestational age. For example, an infant born 4 months prematurely is typically treated with leptin for 4 months or until lung function is restored. For
10 individuals that develop respiratory distress after birth, the individual is treated with leptin for a period of time until lung function is restored, as ascertained by clinical measurements that are known to those skilled in the art.

The leptin compound may be administered subcutaneously, intradermally, intravenously, intramuscularly, intraperitoneally, via enteral tube feeding, via pulmonary delivery, via intranasal
15 delivery, transdermally, orally, via controlled release, via pump, or by any other conventional route of administration for polypeptide drugs. Typically, the leptin compound will be administered continuously during the period of administration, i.e., being delivered at least once per day or via controlled release techniques, such as via transdermal patches or leptin in milk fat globules. Furthermore, leptin may be administered antenatally to those mothers at increased risk
20 of delivering prematurely. Leptin may also be administered antenatally with glucocorticoids. The administration of leptin has been described in U.S. Patent application serial number 09/302117 which is incorporated herein by reference in its entirety.

Leptin may be derived from any mammal. Preferably human leptin is used for the treatment of humans. A leptin compound may include, but is not limited to, either the full active peptide or a biologically active fragment that is capable of binding to the leptin receptor and eliciting a biological effect such as increased surfactant production. Methods for purification of leptin, production of the recombinant form, and active biological fragments have been described in U.S. Patent Nos. 5,552,524; 5,552,523; 5,552,522; 5,521,283; 5,590,830, incorporated herein by reference in their entireties.

In some embodiments, the invention provides compositions for administration which comprises a solution of leptin dissolved or suspended in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, e.g., water, buffered water, 0.8% saline, 0.3% glycine, hyaluronic acid and the like. These compositions may be sterilized by conventional, well-known sterilization techniques, or may be filter-sterilized. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjustments and buffering agents, adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

The concentration of leptin in the pharmaceutical formulations can vary widely, i.e., from less than about 0.1% to as much as 20% to 50% or more by weight, and will be selected primarily by fluid volumes, viscosities, etc., in accordance with a particular mode of administration selected. A value of about 2% is common for many formulations, for example.

For solid compositions, conventional nontoxic solid carriers may be used which include for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating
5 any of the normally employed excipients, such as those carriers previously listed, and generally 10% to 95% of active ingredient, that is one or more leptin compounds of the invention, and more preferably at a concentration of 25% to 75%.

For aerosol administration, leptin is preferably supplied in finely divided form along with an aerosol surfactant and propellant. Typical percentages of leptin are 0.01% to 20% by weight
10 preferably 1%-10%. The aerosol surfactant must, of course be nontoxic, and preferably suitable to the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, oleosteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. The aerosol surfactant may
15 constitute 0.1% to 20% by weight of the composition, preferably 0.25-5%. The balance of the composition is ordinarily propellant. A carrier can also be included, as desired, as with, e.g., lecithin for intranasal delivery.

The leptin compositions of the invention can additionally be delivered in a controlled release system encapsulated form, or an implant by techniques well known in the art. The
20 compositions of the invention can also be delivered via a pump, such as a minipump, or by administration of milk fat globules containing leptin as disclosed in co-pending U.S. patent application number 09/302,117.

Examples

1. Effects of leptin on surfactant production in the fetal lung explant model

We have used a fetal lung explant model to mimic the conditions of the premature lung. Lungs from d21 rat fetuses, term being 22 days, were dissected free of heart, trachea and bronchi and placed in ice-cold serum free Waymouth medium and cut into 1 mm³ pieces on a McIlwain tissue chopper. The lung explants were placed in tissue culture dishes that were scratched (along each half) to facilitate attachment of explants. The excess medium was aspirated and fresh medium (2 ml for 60 mm plate) was gently placed on the explants. The 17-21 d explants were incubated in 95% O₂-5% CO₂, since incubation of these explants in 5% CO₂ in air can cause compression of airways (Gross and Wilson, J. Appl. Physiol. 55: 1725-32, 1983). The petri dishes were placed on a tilting platform and allowed to rest for 90 min in a humidified atmosphere in a CO₂ incubator. Thereafter, the plates were tilted at 3-4 cycles per minute so that during each cycle, one half of the petri dish was exposed to gas phase and the other half was covered with the medium. Leptin at either 1 or 10 ng/ml was added to the explant cultures on the day of establishment of the culture (day 0). The explants were cultured for varying periods of time for up to 3 days, and protein extracts were prepared to establish a time course for leptin effects on the fetal lung. Surfactant proteins were separated by electrophoresis on 15 % polyacrylamide – SDS gels, and transferred to nitrocellulose membranes by electroblotting. The membranes were blocked with 2% gelatin (BioRad), treated overnight with anti-SP-B, and then with secondary antibody (goat anti-rabbit HRP-conjugated antibody). The blots were then reacted with a chemiluminescent substrate solution (SuperSignalR, Pierce Chemical Co.) and exposed to X-ray films to detect proteins that are recognized by the primary antibody. A set of

known concentrations of protein was run in parallel to ascertain that the amount of sample protein is within the linear range of density.

Figure 1 shows that 1 ng/ml leptin increases SP-B production above that of the control cultures after either 48 or 72 h of leptin exposure. A higher concentration of leptin (10 ng/ml) increases SP-B production after 24 h, but decreases SP-B levels after 48 and 72 h.

To explore the effect of gestational age on surfactant production in response to leptin, we exposed younger lung explant cultures (d17) to 1 ng/ml leptin. Total RNA was extracted by homogenizing the explants in 4M guanidine thiocyanate, applying the lysate on a Qiagen RNeasy column (Qiagen, Chatsworth, CA), and recovering total RNA according to the manufacturer's instructions. RNA was quantified by measurement of absorbancy at 260 nm (A_{260}). The quality of RNA was assessed by the A_{260}/A_{280} ratio and by separation on agarose gels. Total RNA (1 μ g) was brought up to 10 μ l in DEPC-treated water. The sample was heated to 75° C for 3 min, placed on ice, and cDNA synthesis was performed by reverse transcription for 15 min at 42° C in a 20 μ l reaction containing 1X PCR buffer II (Perkin-Elmer), 5 mM $MgCl_2$, 1.25 mM each dNTP, 1 U/ μ l RNasin (Promega), 12.5 μ g/ μ l oligo (dT) 15, and 2.5 U/ μ l AMV reverse transcriptase (Promega Madison, WI). Subsequent amplification of the cDNA sequence was performed with 10 μ l of the reverse transcription reaction in 1X Taq buffer, 5% DMSO, 25 pmol each primer (Table 1), and 1.25 U Taq polymerase in a 50 μ l reaction volume.

Table 1: Sequence of PCR primers used in the RT/PCR experiments

Primer	Gene	Sequence
RSPAF	Rat SP-A	5'CCTCTTCTTGACTGTTGTCGCTGG3'
RSPAR	Rat SP-A	5'GCTGAGGACTCCCATTGTTTGCAG3'
RSPBF	Rat SP-B	5'GGAGCTAATGACCTGTGCCAAGAG3'
RSPBR	Rat SP-B	5'CTGGCCCTGGAAGTAGTCGATAAC3'
RSPBR2	Rat SP-B	5'AAGTACTGTGTAACGCTCAGCCAG3'
RSPCF	Rat SP-C	5'GATGGAGAGCCCACCGGATTACTC3'
RSPCR	Rat SP-C	5'GAACGATGCCAGTGGAGCCAATAG
ROBRaF	Rat OB-Ra	5'AGTGAATGCTGTGCAGTCACTCAG3'
ROBRaR	Rat OB-Ra	5'CAAAGAGTGTCCGCTCTCTTTTGG3'
ROBRbF	Rat OB-Rb	5'GGATGAGTGTGAGAGTCAACCCTC3'
ROBRbR	Rat OB-Rb	5'CAGTTCCAAAAGCTCATCCAACCC3'
ACTF1	Rat β -actin	5'TGTATGCCTCTGGTCGTACCAC3'
ACTR1	Rat β -actin	5'ACAGAGTACTTGCGCTCAGGAG3'
GAPDHF	Rat GAPDH	5'GGTCGGTGTCAACGGATTTG3'
GAPDHR	Rat GAPDH	5'GAGATGATGACCCTTTTGGC3'

For assessment of the relative levels of SP-A, SP-B and SP-C transcripts, a multiplex

- 5 RT/PCR reaction with β -actin was used. The temperature profile for the PCR reactions consisted of a 2 min melting step at 95° C, then 30 cycles of 30 s at 94° C, 30 s at 55° C, and 60 sec at 65° C, followed by a final extension step of 5 min at 72° C. RT-PCR products were separated by size on a 4% agarose gel and stained with ethidium bromide. Gel visualization and quantitative analysis of relative band intensities was performed using Eagle Eye II hardware and
- 10 software (Stratagene, La Jolla, CA).

Figure 2 shows that leptin significantly increases SP-A and SP-C mRNA levels.

However, leptin also increases the levels of β -actin in fetal lung explant cultures. This results in an underestimation of the actual increase in surfactant mRNA levels. Therefore, we chose to use 18S rRNA to determine the relative levels of surfactant mRNA because of the invariant

expression of 18S rRNA across tissues and treatments. Since 18S rRNA is much more abundant than any mRNA species, modified 18S rRNA primers called competitors (Ambion) are used that cannot be extended by Taq polymerase. By adjusting the ratio of competitors to normal 18S rRNA primers, the RT/PCR signal for 18S rRNA can be decreased to the level of even rare messages, as described by the manufacturer. Figure 3 shows the results of such an experiment for determining the relative levels of the surfactant mRNAs to 18S rRNA. Figure 3 shows that leptin increases the mRNA levels for SP-A, SP-B, and SP-C relative to the levels of 18S rRNA by 1.6-, 5-, and 2-fold, respectively, in d17 lung explant cultures after 3 days in culture. These experiments support the hypothesis that leptin has an effect on the maturation of type II alveolar cells.

2. Changes in leptin receptor gene expression in relation to lung maturation

Both the long (OB-Rb) and short (OB-Ra) forms of the leptin receptor are expressed in fetal lung explant cultures (Figure 4). As the fetal lung cells mature in culture, the expression of *OB-Ra* mRNA increases, whereas *OB-Rb* mRNA levels decrease. Leptin administration similarly decreases expression of *OB-Rb* mRNA, whereas dexamethasone increases *OB-Rb* mRNA levels in fetal lung explants (Figure 4). Taken together, these data demonstrate the presence of *OB-Ra* and *OB-Rb* mRNA in rat fetal explant lung cultures. As type II alveolar cells mature in culture, mRNA levels of *OB-Ra* increase and *OB-Rb* decrease; leptin administration further decreases *OB-Rb* mRNA levels, whereas dexamethasone increases *OB-Rb* mRNA expression.

3. Leptin increases mRNA levels of surfactant proteins in isolated fetal alveolar type II cells

To determine if the leptin effect on surfactant production was due to a direct effect of leptin on the type II alveolar cell, we determined whether the effects of leptin could be

reproduced in isolated type II alveolar cells in culture. Alveolar type II cells were obtained from the lungs of 19-d gestation fetal rats by the method described by Bhandari et al., (Pediatr. Res. 41:166-71, 1997). In brief, lungs of 19-day gestation fetal rats were removed, dissected free of connective tissue and nonparenchymal pulmonary tissue, and cultured as explants for 40-48 h in serum free Waymouth MB 752/1 medium with penicillin and streptomycin in humidified 95%O₂/5%CO₂ at 37°C. During this time, endothelial and blood cells do not survive, which is a crucial step in the enrichment of primary cultures of Type II cells from fetal lung. The explant cells were then harvested and the cells dissociated using a solution of collagenase, trypsin and DNase. The mixed cell suspension was subjected to three differential adhesions to remove fibroblasts. The non-adherent suspension containing an enriched population of fetal type II cells was plated at 2 x 10⁶ cells/35 mm dish in 2 ml of minimum essential medium containing penicillin (100 U/ml), kanamycin (100 µg/ml) and 2% fetal bovine serum. The cells were cultured for 20-22 h at 37°C in 5%CO₂ /room air (Bhandari et al., Pediatr. Res. 41:166-71, 1997). Cultures contain 90-95% type II cells of which >99% are viable as determined by exclusion of the vital dye, trypan blue. The usual yield of type II cells from the lungs of the fetuses (10-16) per pregnant rat is approximately 10⁷ cells.

Type II cells from d19 fetal lungs were cultured for 24 h and then exposed to either 1 or 10 ng/ml leptin for 24 h. Total RNA was isolated from these cells and then examined for SP-A, SP-B, SP-C, and GAPDH (housekeeping gene) mRNA expression. Figure 5 shows that a 24 h treatment with 1 ng/ml leptin increased SP-A, SP-B, and SP-C, but not GAPDH, mRNA levels after 24 h. These data suggest that leptin acts directly on the type II cell and that the effects of leptin on SP-A, SP-B, and SP-C mRNA levels are most likely exerted at the transcriptional level.

Taken together, these data demonstrate that leptin administration both in fetal lung explant cultures and isolated type II alveolar cultures results in increases in SP-A, SP-B, and SP-C mRNA. Lung explants from day 21 fetuses produced increased surfactant proteins with leptin administration, possibly indicating an effect on maturation of type II alveolar cells. Thus, leptin provides a means to increase surfactant production in immature lungs, ultimately resulting in an additional treatment modality for premature infants with RDS and for other conditions characterized by insufficient surfactant production.

4. Antenatal treatment of pregnant rats with leptin

Leptin (1 mg/kg body weight) was administered to pregnant rats at d16 of gestation and 24 h later by intraperitoneal injection. Dexamethasone at 6 mg per kg body weight was administered at similar time intervals as leptin. In addition, the effect of a combination of leptin and dexamethasone treatment was tested. After 48 h of leptin, dexamethasone or leptin/dexamethasone exposure, premature delivery of the rat pups was induced at d18, which is similar to 30 weeks of gestation in the human. The fetuses from each litter were pooled and weighed, and various tissues from both the rat fetuses and mothers were also dissected and weighed. Table 2 shows that antenatal treatment with leptin increased the average weight of the fetal lungs in relation to their body weight by 51%. Antenatal treatment with dexamethasone increased fetal lung weight by 41%. Interestingly, combined therapy with leptin and dexamethasone increased fetal lung weight by 62%.

Table 2: Effect of antenatal treatment with dexamethasone, leptin, or dexamethasone and leptin on weight of maternal and fetal lungs

	Control	Dexamethasone	Leptin	Dex + Leptin
Maternal lung weight (g)	1.07	0.99	1.21	1.16
Average weight fetal lungs (g)	0.61 (n=9)	0.79 (n=9)	0.87 (n=9)	0.95 (n=9)
Average weight fetal head (g)	0.47 (n=9)	0.43 (n=9)	0.45 (n=9)	0.47 (n=9)
Average weight fetus (g)	1.64 (n=9)	1.52 (n=9)	1.56 (n=9)	1.59 (n=9)
Weight fetal lung (g)/g fetus	0.37	0.52	0.56	0.60

Histologic analysis of the lung tissue was done to determine the basis for the increased fetal lung weight. Type II cells were identified by alkaline phosphatase staining as previously described (Post and Smith, Am. Rev. Respir. Dis. 137: 525-30, 1988). Unfixed frozen 5 μ m sections of fetal lung tissue were stained histochemically with alkaline phosphatase at pH 8.74 to identify type II alveolar cells. Slides were incubated for 60 minutes at 37°C in 25 ml of 0.2M Tris-HCl buffer and 25 ml of deionized water, 5 mg of Naphthol AS-BI phosphate, 0.1 ml dimethylformamide, and 30 mg of fast red TR. The slides were then rinsed in 3 changes of deionized water, counterstained for 30 seconds in Harris hematoxylin and blued in running water for 3-5 minutes. Slides were mounted from water in Advantage™. The histologic analysis revealed that the increase in fetal lung weight in both the leptin- and dexamethasone-treated rats was paralleled by an increase in the number of type II alveolar cells (Figure 6).

It will be readily understood by those persons skilled in the art that the present invention is susceptible to broad utility and application. Many embodiments and adaptations of the present invention other than those herein described, as well as many variations, modifications

and equivalent arrangements, will be apparent from or reasonably suggested by the present invention and foregoing description thereof, without departing from the substance or scope of the invention.

Accordingly, while the present invention has been described here in detail in relation to its preferred embodiment, it is to be understood that this disclosure is only illustrative and exemplary of the present invention and is made merely for the purposes of providing full and enabling disclosure of the invention. The foregoing disclosure is not intended to be construed or to limit the present invention or otherwise to exclude any other such embodiments, adaptations, variations, modifications and equivalent arrangements, the present invention being limited by the claims and the equivalents thereof.

Claims

What is claimed is:

1. A method for improving lung surfactant production in an individual with impaired surfactant production, the method comprising administering a leptin compound to the individual for a time and in an amount sufficient to enhance surfactant production.

2. The method of Claim 1, wherein the individual is a mammal.

3. The method of Claim 1, wherein the individual is an infant with an in utero development of less than nine months.

4. The method of Claim 1, wherein the leptin compound comprises at least a biologically active fragment of leptin.

5. The method of Claim 1, wherein the leptin compound is a recombinant protein comprising at least a biologically active fragment of leptin.

6. The method of Claim 1, wherein the leptin compound is administered in a dosage from about 0.1 ng/kg body weight to about 100 mg/kg body weight.

7. The method of Claim 1, wherein the leptin compound is administered by a method selected from the group consisting of subcutaneously, intradermally, intravenously,

intramuscularly, intraperitoneally, transdermally, orally, enteral tube feeding, pulmonary delivery, intranasal delivery, controlled release delivery and pump delivery.

8. The method of claim 1 wherein the step of administering the leptin compound
5 includes administering to the individual milk fat globules containing leptin.

9. A method for improving lung surfactant production in an individual with respiratory distress syndrome, the method comprising administering a leptin compound to the individual for a time and in an amount sufficient to enhance surfactant production.

10
10. The method of Claim 9, wherein the leptin compound comprises at least biologically active fragment of leptin.

11. The method of Claim 9, wherein the leptin compound is administered in a dosage
15 from about 0.1 ng/kg body weight to about 100 mg/kg body weight.

12. The method of Claim 9, wherein the leptin compound is administered by a method selected from the group consisting of subcutaneously, intradermally, intravenously, intramuscularly, intraperitoneally, transdermally, orally, enteral tube feeding, pulmonary
20 delivery, intranasal delivery, controlled release delivery, and pump delivery.

13. The method of claim 9 wherein the step of administering the leptin compound includes administering to the individual milk fat globules containing leptin.

14. A method for improving lung surfactant production in an individual with
5 Bronchopulmonary Dysplasia, the method comprising administering a leptin compound to the individual for a time and in an amount sufficient to enhance surfactant production.

15. The method of Claim 14, wherein the leptin compound comprises at least a biologically active fragment of leptin.

10 16. The method of claim 14 wherein the step of administering the leptin compound includes administering to the individual milk fat globules containing leptin.

ABSTRACT OF THE DISCLOSURE

The invention provides a method for treating infants, children or adults suffering from pulmonary distress caused by low or insufficient production of surfactant. It is particularly suitable for treating premature infants suffering from Respiratory Distress Syndrome. The method comprises administering a leptin compound to an individual with impaired surfactant production for a time and in an amount sufficient to enhance surfactant production. The method may be used for treatment of any mammal with impaired lung surfactant production.

FIGURE 1

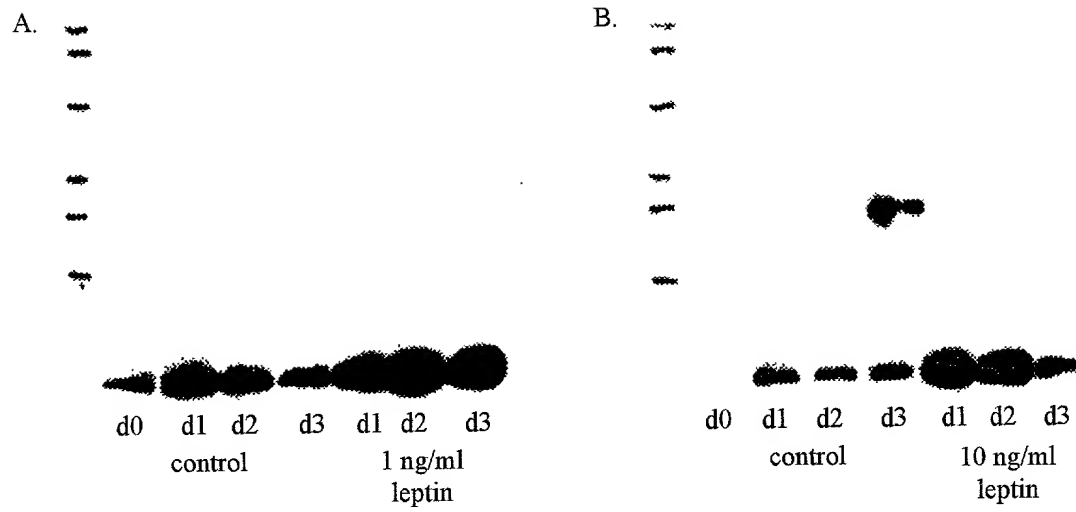


FIGURE 2

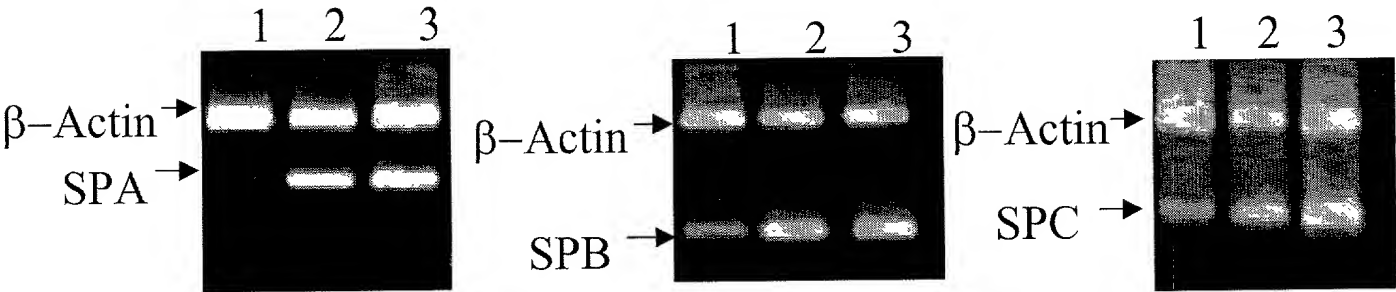


FIGURE 3

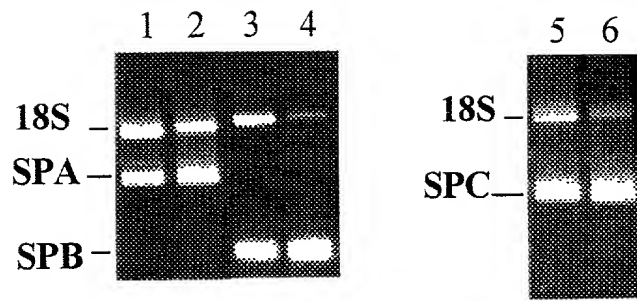


FIGURE 4

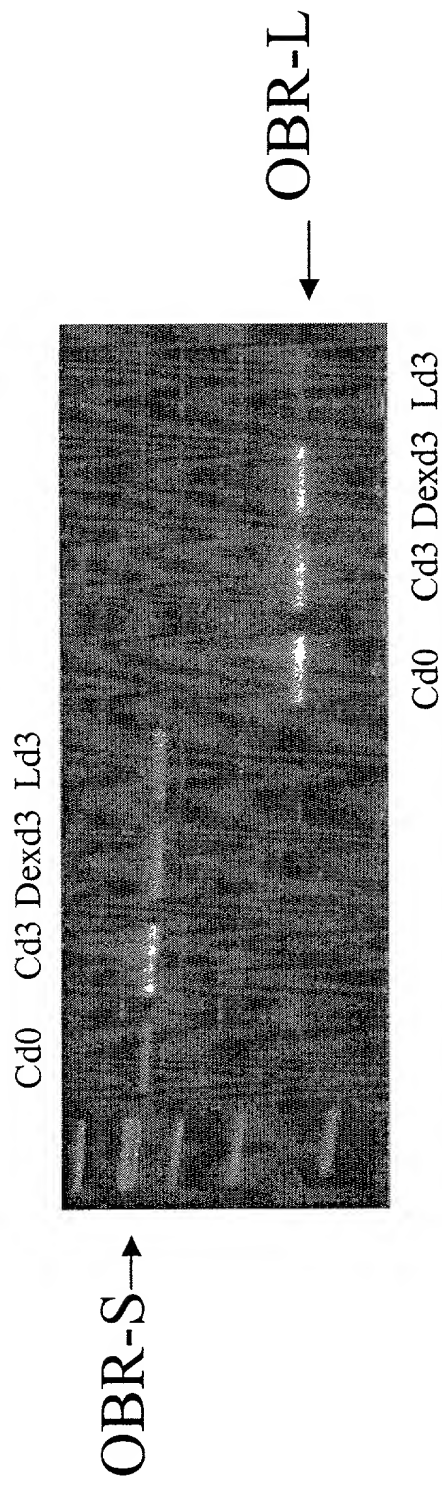


FIGURE 5

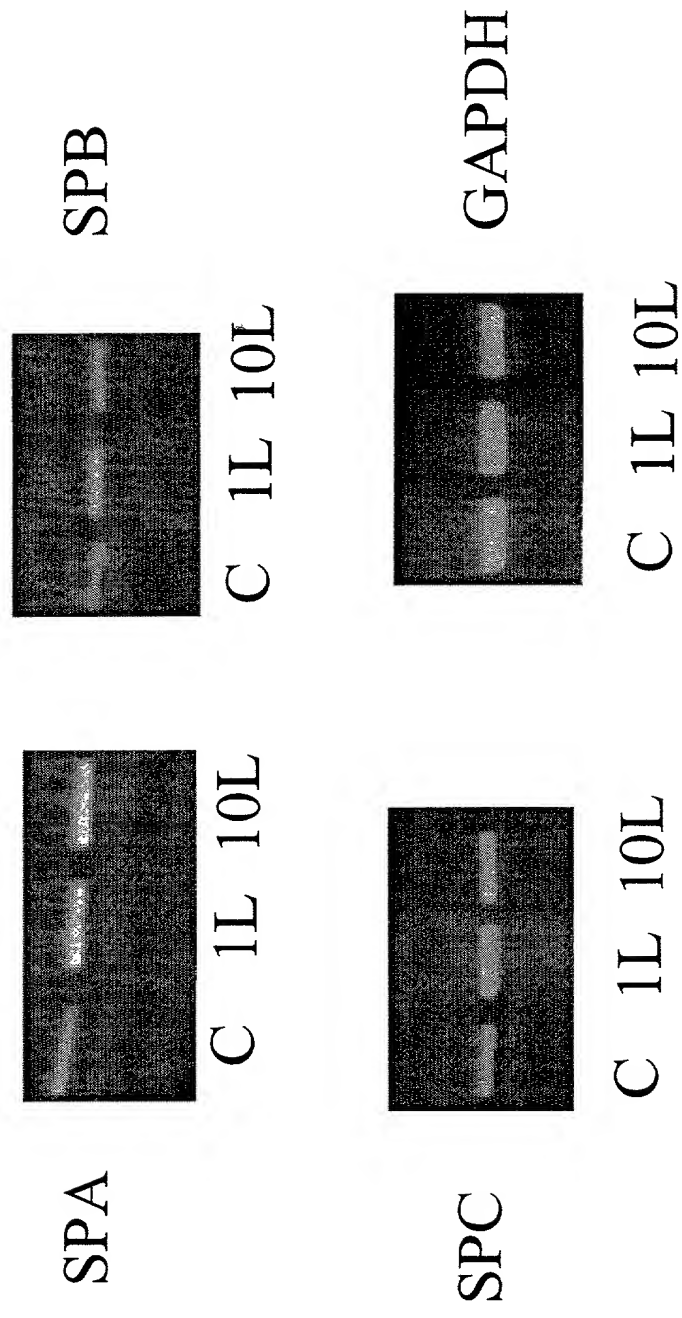
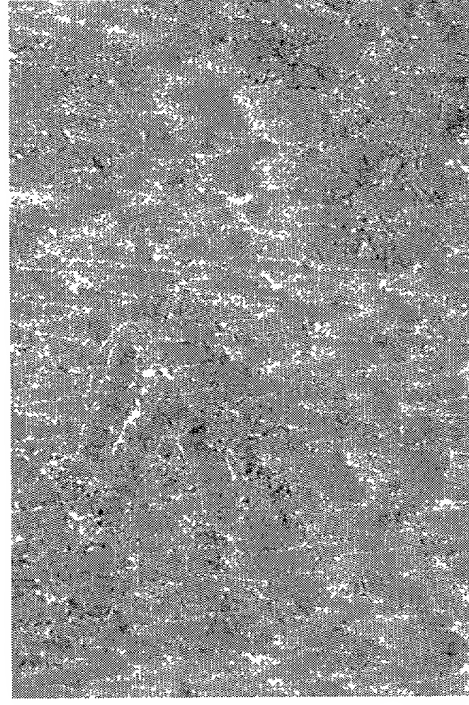
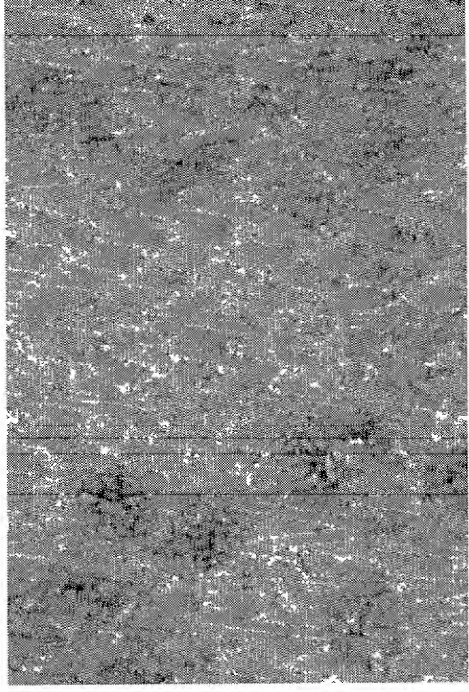


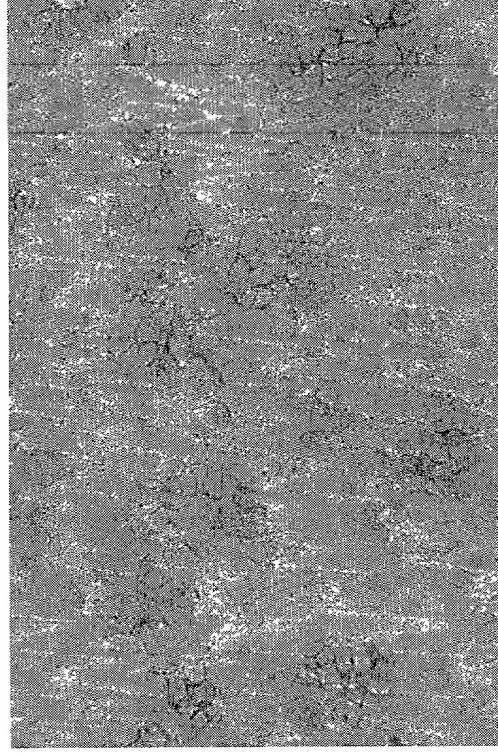
FIGURE 6



Control



Dexamethasone



Leptin

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name;

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

METHOD FOR TREATING RESPIRATORY DISTRESS SYNDROME

the specification of which:

(check one) ☒ is attached hereto
☐ was filed on
as Application Serial No.
and was amended on _____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1.56*

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)			priority claimed	
(Number)	(Country)	(Day/Month/Year Filed)	yes	no
_____	_____	_____	_____	_____
(Number)	(Country)	(Day/Month/Year Filed)	yes	no

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

_____	_____	_____
(Application Serial No.)	(Filing Date)	(Status: patented, pending, abandoned)

and any continuation applications thereof currently pending.

Power of Attorney: As a named inventor, I hereby appoint C. Lamont Whitham, Reg. No. 22,424, Marshall M. Curtis, Reg. No. 33,138, Michael E. Whitham, Reg. No. 32,635, Philip D. Lane, Reg. No. 41,140 and Nancy J. Jensen, Reg. No. 45,912, as attorneys and/or agents to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. All correspondence should be directed to McGuireWoods, One James Center, 901 East Cary Street, Richmond, Virginia 23219-4030. Telephone calls should be directed to Nancy J. Jensen, Ph.D. at McGuireWoods at 804-775-1174.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full Name of Sole

or First Inventor Vicky L. Funanage, Ph.D.Inventor's Signature Vicky L. Funanage, Ph.D. Date 11/13/00Residence 2403 Landon Drive, Wilmington, Delaware 19810Citizenship United StatesPost Office Address Same as above

Full Name of Joint

or Second Inventor Sandra G. Hassink, M.D.Inventor's Signature Sandra G. Hassink MD Date 11/13/00Residence 2602 Pennington Drive, Wilmington, Delaware 19810Citizenship United StatesPost Office Address Same as above

Full Name of Joint

or Third Inventor Susan M. Kirwin, B.S.Inventor's Signature Susan M. Kirwin Date 11/13/00Residence 4 Heatherstone Way, Thornton, Pennsylvania 19373Citizenship United StatesPost Office Address Same as above

Full Name of Joint

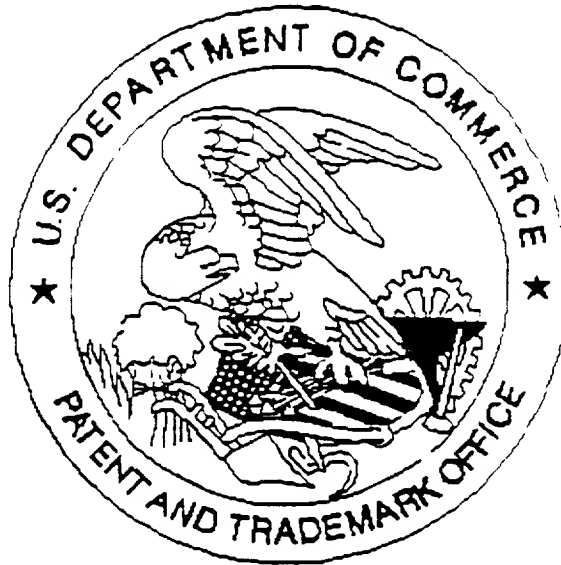
or Fourth Inventor Darlise O'Connor, B.S. B.A.Inventor's Signature Darlise O'Connor Date 11/13/00Residence 103 Jupiter Road, Newark, Delaware 19711Citizenship United StatesPost Office Address Same as above

Title 37, Code of Federal Regulations, § 1.56:

(a) A patent by its very nature is affected with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith toward the Patent and Trademark Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is canceled or withdrawn from consideration, or the application becomes abandoned.

(b) Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and (1) it establishes, by itself or in combination with other information, a prima facie case of unpatentability; or (2) it refutes, or is inconsistent with, a position the applicant takes in: (i) opposing an argument of unpatentability relied on by the Office, or (ii) asserting an argument of patentability.

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